The Effects of Strontium-Substituted Bioactive Glasses on Osteoblasts and Osteoclasts in Vitro

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Abstract

Bioactive glasses (BG) which contain strontium have the potential to combine the known bone regenerative properties of BG with the anabolic and anti-catabolic effects of strontium cations. Here we created a BG series (SiO_2-P_2O_5-Na_2O-CaO) in which 0 to 100% of the calcium was substituted by strontium and tested their effects on osteoblasts and osteoclasts *in vitro*. We show that ions released from strontium-substituted BG enhance metabolic activity in osteoblasts. They also inhibit osteoclast activity by both reducing tartrate resistant acid phosphatase activity and inhibiting resorption of calcium phosphate films in a dose-dependent manner. Additionally, osteoblasts cultured in contact with BG show increased proliferation and alkaline phosphatase activity with increasing strontium substitution, while osteoclasts adopt typical resorption morphologies. These results suggest that similarly to the osteoporosis drug strontium ranelate, strontium-substituted BG may promote an anabolic effect on osteoblasts and an anti-catabolic effect on osteoclasts. These effects, when combined with the advantages of BG such as controlled ion release and delivery versatility, may make strontium-substituted BG an effective biomaterial choice for a range of bone regeneration therapies.

Keywords: Bioactive glass, osteoblast, osteoclast, bone regeneration, osteoporosis

Running title: Strontium-substituted bioactive glasses

1. Introduction

Osteoporosis is common among postmenopausal women and is marked by an imbalance in the normal process of bone remodelling. In osteoporotic bone, osteoclasts resorb too much bone while osteoblasts fail to create enough, which together disrupt the bone microarchitecture leaving it porous and prone to fracture. Annual direct costs due to osteoporosis-related fracture in the United States are estimated in the range of US$20 billion [1] and its disease burden in Europe is estimated to be greater than that attributed to any cancer save lung [2]. Strontium ranelate (SrR), sold under the trade name Protelos®, is a drug approved for the treatment and prevention of osteoporosis and has been shown to reduce the number of hip and vertebral fractures in post-menopausal women [3, 4]. The drug combines two atoms of stable strontium with the organic moiety ranelic acid. The cellular mode of action of SrR appears to be through the strontium cations themselves, which have been shown to work both *in vivo* and *in vitro* by stimulating osteoblasts to make new bone and preventing osteoclasts from resorbing bone [5, 6]. That is, data suggest that SrR works by helping resident cells in the bone to restore the normal bone remodelling balance.

Bioactive glasses (BG) form a strong bond with living tissue *via* the formation of a hydroxyapatite layer on their surface [7] and have been used to repair hard tissues in a variety of craniofacial, maxillofacial, and periodontal applications [8]. BG particulate, for example, is used in a variety of dental procedures [9], and many BG compositions can be formed into scaffolds for tissue engineering [10]. Surface reactivity, however, is not their only mechanism of action as BG also release ions that promote the

osteoblast phenotype [11, 12]. *In vitro* studies have demonstrated that BG stimulate osteoprogenitors to differentiate to mature osteoblasts that produce bone-like nodules [13, 14]. Similarly, *in vivo* studies have shown gradual degradation of implanted BG with subsequent formation of new bone at the implant surface [15, 16]. Because of similarities in their charge and ionic radius, strontium can potentially be substituted for calcium in BG, creating a material that may combine the bone bonding and osteoblast stimulating properties of BG with the bone anabolic and anti-catabolic properties of SrR, and may act as an effective material for bone regeneration applications.

Here we created a series of BG (SiO_2-P_2O_5-Na_2O-CaO) in which strontium was substituted for calcium on a mole percentage basis. We then tested their effects on cells cultured both in contact with the BG and treated with culture media containing ions released from the BG as they dissolved. The effects of strontium substitution in the BG were tested on osteoblast and osteoclast cultures and demonstrate that strontium-substituted BG promote osteoblast proliferation and activity and decrease osteoclast activity and resorption. As BG are a well-studied biomaterial and excellent system for controlled ion release, strontium-substituted BG may find use in a range of bone regeneration applications including as porous scaffolds, rigid materials and particulate.

2. Materials and Methods

2.1 Bioactive glass synthesis

BG containing 46.46SiO_2 – 1.07 P_2O_5 – 26.38 Na_2O – 23.08 (SrO:CaO) (mole %) where either no calcium (0%) or 10, 50 or 100% of the calcium was substituted for with strontium were produced by a melt-quench route as previously described [17]. Briefly, the composition was melted in a platinum crucible at 1400 °C for 90 minutes and rapidly quenched in deionised (dH_2O) water. BG frits were then ground and sieved to yield powders <38 μm in diameter. Some frits were also re-melted at 1450 °C and poured into pre-heated graphite moulds held just above the glass transition temperature (which varied between 450 and 500 °C depending on the percent strontium substitution [17]) to obtain rods 10 mm in diameter. Discs 1 mm thick were cut from the rods with a low speed diamond saw (Buehler IsoMet®, Dusseldorf, Germany) using isopropanol as a coolant.

2.2 Dissolution ion studies

2.2.1 Dissolution ion media

1.5 g/L of BG powder was added to RPMI 1640 or Dulbecco's modified Eagle's (DMEM) cell culture medium (Invitrogen, Paisley, UK), incubated on a roller at 37 °C for 4 hours and then passed through a 0.2 μm filter. Samples of dissolution ion media were collected after 10, 30, 60, 120, and 240 minutes for elemental analysis. Media were supplemented as described in section 2.2.3 and equilibrated in a 37 °C/5% CO_2 incubator overnight before being placed on cells.

2.2.2 Elemental analysis

Cell culture media samples containing dissolution ions from BG were diluted by a factor of 10 in dH_2O and elemental concentrations of Ca, P, Si and Sr ions were measured on an inductively coupled plasma – optical emission spectrometer (ICP-OES) (iCAP 6000, Thermo Scientific, Waltham, MA, USA).

2.2.3 Cell culture with dissolution ions

The human osteosarcoma cell line, Saos-2, was obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and routinely cultured under standard conditions (37 °C, 5% CO_2/95% air, 100% humidity) in RPMI 1640 media supplemented with 10% (v/v) foetal bovine serum (FBS) and 2mM L-glutamine (all reagents from Invitrogen). For experiments, cells were plated at 30,000 cells/cm^2 and cultured for up to 28 days.

The mouse monocyte cell line, RAW264.7, which differentiates to osteoclasts under appropriate conditions, was obtained from American Type Culture Collection (ATCC, Teddington, UK) and cultured in DMEM containing 4.5 g/L glucose and 1.5 g/L sodium bicarbonate (Sigma-Aldrich, Dorset, UK) supplemented with 10% (v/v) FBS and 4 mM L-glutamine. The nitrogenous bisphosphonate pamidronate (Sigma), a drug known to prevent osteoclast-mediated bone resorption *in vivo* [18], was added at a concentration of 1 μM as a control. For erosion pit studies, an additional control was created by adding 1mM $SrCl_2 \cdot 6H_2O$ (Sigma) to standard cell culture medium. To initiate differentiation, 20 ng/mL soluble murine Receptor Activator for Nuclear Factor κ B Ligand (RANKL, PeproTech EC, London, UK) was added. For

experiments, cells were plated at 30,000/cm^2 (unless stated otherwise) and cultured for up to 6 days.

2.2.4 Metabolic activity of cells

Cell metabolic activity was measured using the tetrazole MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma), which is reduced to formazan by mitochondria in metabolically active cells. 50 μL of a 5 mg/mL solution of MTT in phosphate buffered saline (PBS) was added to the culture media of live cells at indicated time points. After 2 hours, media was removed and the formazan was solubilised in dimethyl sulfoxide. The absorbance of the resulting solution was read on a colorimetric plate reader at 592 nm.

2.2.5 Actin staining of cells

Differentiated RAW264.7 cultures were fixed for 15 minutes in 4% (w/v) paraformaldehyde in dH_2O. After fixation, cultures were washed in PBS, permeabilised in 1% (v/v) Triton X-100 in dH_2O for 5 minutes, washed again and then stained for 20 minutes with AlexaFluor 568 Phalloidin (Invitrogen; 5 U/mL in 1% (w/v) bovine serum albumin (BSA) in PBS) to visualise the actin cytoskeleton. Cultures were then rinsed with 1% (w/v) BSA in PBS and DAPI was added to stain cell nuclei. Cultures were then imaged using an epifluorescent microscope.

2.2.6 Tartrate resistant acid phosphatase (TRAP) activity and total cell quantification

TRAP activity, a marker of osteoclast differentiation and resobing activity [19], was measured in RAW264.7 cells by quantifying the conversion of p-nitrophenyl phosphate to p-nitrophenyl in the presence of sodium tartrate under acidic conditions. After 2, 4, or 6 days in culture, cells were lysed in dH_2O by three freeze-thaw cycles and 1 mg/mL p-nitrophenyl phosphate in 0.1 M glycine buffer with 0.1 mM $ZnCl_2$, 0.1 mM $MgCl_2$ and 20 mM sodium tartrate (pH = 5.0) was added to the lysates. The reaction was stopped with 1 M NaOH and the absorbance read at 405 nm on a colorimetric plate reader. TRAP activity is expressed as nmoles p-nitrophenyl liberated per minute. The total number of cells in the same lysates was determined by quantifying the activity of the stable cytosolic enzyme lactate dehydrogenase with a Cytotox96® Non-Radioactive Cytotoxicity Assay (Promega, Southampton, UK), according to the manufacturer's instructions.

2.2.7 Erosion pit formation by osteoclasts

The formation of erosion pits by differentiated RAW264.7 cells was evaluated using BioCoat™ Osteologic™ (BD Biosciences Europe, Erembodegem, Belgium) chamber well slides which are coated with a thin calcium phosphate (CaP) layer suitable for cell culture. 9600 cells/well were cultured for 4 days in the presence of 20 ng/mL RANKL with either no additional supplements, 1 mM $SrCl_2 \cdot 6H_2O$, 1 μM pamidronate, or BG dissolution ion media. Cells were removed with sodium hypochlorite and slides stained with silver nitrate according to the manufacturer's instructions. Micrographs of stained wells were taken on an inverted light microscope

and the eroded area was determined using the histogram function in ImageJ (freeware from the NIH).

2.3 Bioactive glass discs studies

2.3.1 Cell culture on bioactive glass discs

BG discs were sterilised under UV light for 2 hours per side and preconditioned for three days in RPMI 1640 medium (exchanged every day). 30,000 Saos-2 cells/cm^2 were seeded on BG discs and cultured for 1, 7, or 14 days in standard culture medium, which was exchanged three times per week. 50,000 RAW264.7 mouse monocytes/cm^2 were also seeded on 0% strontium BG discs and differentiated for 4 days in standard medium supplemented with 50 ng/mL RANKL to visualise osteoclast differentiation.

2.3.2 Scanning electron microscopy (SEM) of osteoclasts

After 4 days, RAW264.7 cells on 0% BG discs were prepared for SEM imaging to view osteoclasts. Discs were washed twice in PBS and fixed in 2.5% (v/v) glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.3) for 40 minutes at 4 °C. Samples were then washed twice in PBS and dehydrated in a graded ethanol series followed by critical point drying with hexamethylsilasane. Following fixation, discs were sputter coated with gold and viewed on a Leo 1525 Gemini SEM operated at 10 kV.

2.3.3 Alkaline phosphatase (ALP) activity and total cell quantification

ALP activity, a marker of osteoblast differentiation and mineralisation, was measured in Saos-2 cultured on BG discs using the substrate p-nitrophenyl phosphate. At indicated time points, cultures were lysed in dH_2O containing 0.9% (v/v) Triton X-100 and reacted with 1 mg/mL p-nitrophenyl phosphate in 0.1 M glycine buffer with 0.1 mM $ZnCl_2$ and 0.1 mM $MgCl_2$ (pH = 10.4). The reaction was stopped with 1 M NaOH and the absorbance read on a colorimetric plate reader at 405 nm. The total number of cells in the same lysates was determined using a Cytotox96® Non-Radioactive Cytotoxicity Assay according to the manufacturer's instructions.

2.4 Statistical analyses

Statistical significance in MTT, TRAP and ALP assays was assessed by ANOVA with post-hoc LSD test to determine statistical significance between individual groups. A two-tailed Spearman's rho rank correlation was used to determine statistical significance in erosion pit area experiments. A p value of les s than 0.05 was considered statistically significant. For experiments on BG discs, n = 6 per composition per time point. Erosion pit area data represent means of 2 experiments and average data from at least 4 wells.

3. Results

3.1 Bioactive glass formation

BG frits and rods were optically clear and were shown to be amorphous by X-ray diffraction [17]. Solid state nuclear magnetic resonance and vibrational spectroscopy further demonstrated that 0, 50 and 100% substituted BG had predominantly Q^2 silicate structures [17].

3.2 Dissolution ions studies

3.2.1 Ion release

The elemental concentrations of Ca, P, Si and Sr released from BG into cell culture media are shown in Figure 1. The dissolution of calcium into both RPMI 1640 and DMEM was dependent on BG composition, with decreasing media calcium concentration with increasing strontium substitution. The phosphorus concentration in RPMI 1640 changed little over the 4 hour release period while in DMEM a general decrease in phosphorus concentration was observed for all BG compositions. The concentration of silicon in both RPMI and DMEM increased with dissolution time and was variable depending on culture media type and BG composition. The concentration of strontium was dependent on BG composition with increasing strontium substitution resulting in higher media strontium concentrations after 4 hours.

3.2.2 Metabolic activity of cells

After 1 and 7 days in culture, there were no significant differences in the MTT activity of Saos-2 cells treated with BG dissolution ions compared to cells in standard

culture medium (Figure 2). After 14 and 28 days in culture, the MTT activity in cells treated with dissolution ions from all compositions of BG was significantly enhanced compared to controls. After 14 days in culture, MTT activity was significantly greater in Saos-2 cells treated with dissolution ions from 10, 50 and 100% substituted BG compared to 0% substituted BG. After 28 days in culture, cells treated with dissolution ions from 100% strontium-substituted BG had significantly higher MTT activity than that in any other group.

3.2.3 Formation of erosion pits by osteoclasts

RAW264.7 cells treated with RANKL formed erosion pits on CaP-coated slides (Figure 3a) while those treated with RANKL and 1 μM pamidronate formed very few erosion pits (Figure 3b). Mean erosion pit area, an indicator of osteoclast activity, decreased when RAW264.7 monocytes were treated with dissolution ions from BG in which an increasing proportion of calcium was substituted for with strontium (Figure 3c). The correlation coefficient between strontium substitution and mean erosion pit area was $r^2 = 0.94$, which was statistically significant by Spearman's rho rank correlation.

3.2.4 Osteoclast differentiation and TRAP activity

RAW264.7 cells differentiated to large multinucleated osteoclasts in the presence of RANKL. Actin staining highlighted typical podosome belts in mature osteoclasts which appeared as prominent rings encasing multiple nuclei (Figure 4a). Cultures treated with RANKL and 1 μM pamidronate (Figure 4b) failed to form osteoclasts

with distinctive podosome belts. After 4 days in culture, cells treated with dissolution ions from 100% strontium-substituted BG showed significantly less TRAP activity when compared to all other BG treated groups (Figure 4c). After 6 days in culture, RAW264.7 treated with dissolution ions from 10%, 50% and 100% strontium-substituted BG all had significantly lower TRAP activity when compared to cells treated with dissolution ions from 0% BG. The presence of 1 μM pamidronate significantly reduced total TRAP activity at all time points compared to all other groups. The presence of dissolution ions from all BG compositions, however, did not have an effect on cell number as no significant differences were detected between any groups at any time point (Figure 4d). When TRAP activity was normalised to cell number, similar trends were noted although ANOVA determined them not be significant with the exception that the group treated with 1 μM pamidronate had significantly lower TRAP activity per cell than any other group at any time point (Supplementary Figure 1).

3.3 Bioactive glass discs studies

3.3.1 Total cell quantification

The total number of Saos-2 cells on BG discs increased over the two week culture period for all BG compositions (Figure 5a). After 24 hours in culture, there were no significant differences between the number of cells on any BG disc composition. However, after 7 days in culture, there were significantly more cells on 100% substituted BG discs than on 0%, and after 14 days in culture, there were significantly

more cells on 50 and 100% strontium-substituted BG discs compared to either 0 or 10% strontium-substituted BG discs.

3.3.2 ALP activity

Saos-2 cells cultured on BG discs showed increased total ALP activity with increasing strontium substitution for calcium (Figure 5b). There were no significant differences in total ALP per disc after 24 hours in culture, but after 1 week, cells on 100% strontium-substituted BG discs had significantly higher total ALP activity than those on 0% or 10% BG discs. After 2 weeks in culture, cells on BG discs with 50 or 100% strontium substitution had significantly higher total ALP activity than those on discs with 0% or 10% strontium substitution. This trend held when ALP activity was normalised per cell number as determined by LDH activity. There were no significant differences after 1 or 7 days in culture, but after 2 weeks, cells on BG discs containing 50 and 100% strontium substitution had significantly higher ALP activity per cell than those on 0% strontium-substituted BG discs (Supplementary Figure 2).

3.3.3 SEM of osteoclasts

RAW264.7 monocytes cultured on standard BG discs differentiated to large cells with apico-basal polarisation, morphology typical of resorbing osteoclasts (Figure 6). Osteoclasts with similar morphologies were observed adherent to the surfaces of all BG compositions by SEM (not shown). Many undifferentiated monocytes were also visible.

4. Discussion

Calcium has been reported to act as a network modifier in BG. As strontium has a similar charge and ionic radius to calcium, when it is substituted into the glass network, it may play a similar role. The lower charge to size ratio of the strontium ion compared to the calcium ion (due to the slighter larger ionic radius of strontium), however, should create an expanded and more loosely cross-linked glass network, but not fundamentally alter the glass structure.

The release of Ca, P, Si and Sr into cell culture medium during incubation with BG is complicated by ions already present in cell culture media, which themselves vary among media formulations. Our ICP-OES data show that despite its release from the BG, the calcium concentration in RPMI 1640 decreases and in DMEM stays fairly constant with incubation time. This is likely due to the formation of a CaP layer on the surface of the BG particles during incubation, which depletes the culture media of calcium [20]. This hypothesis is supported by the decrease in phosphorus concentration over time during incubation of BG in DMEM. The high concentration of phosphorus in RPMI 1640 (174 ppm) provides an excess available to form the CaP layer and so its concentration does not significantly diminish during incubation.

Silicon, which is thought to be responsible for the cell-stimulating activities of BG dissolution ions due its essential role in bone formation [21] is released into culture media over time due to the breakup of the outer silica layers of the BG network [10]. While silicon release into simulated body fluid is reported to happen quickly (30 minutes to 2 hours), release into cell culture media proceeds more slowly [20], and

although our data show that the silicon concentration in cell culture media increases with time for all BG compositions and in both media formulations, it does not reach its solubility limit. As expected, the substitution of strontium for calcium in BG results in increased levels of strontium in both RPMI 1640 and DMEM cell culture media. Taken together, these data suggest that the glass chemistry can be tailored to release an appropriate level of strontium and/or other ions depending on the application.

At early time points, the metabolic activity of Saos-2 cells treated with BG dissolution ions was not significantly different from that observed in cells treated with standard culture medium indicating that the dissolution products of all compositions of BG were non-cytotoxic. However, after 2 weeks in culture, dissolution ions from all formulations of BG enhanced total metabolic activity in Saos-2 cells compared controls. This has been previously shown in primary human osteoblasts treated with 45S5 Bioglass® dissolved in DMEM, which showed increased proliferation compared to controls [22]. We also noted that MTT activity in cells treated with strontium-substituted BG was significantly enhanced compared to non-strontium-containing groups. This suggests that dissolution ions released from the strontium-substituted BG further enhance metabolic activity in Saos-2 beyond that caused by the presence of dissolution ions from standard BG. This is in agreement with previous studies which have shown that SrR promotes osteoblast proliferation [23] and suggests that strontium ions may act synergistically with other ions (most likely silicon) released from BG.

Treating RAW264.7 monocytes with dissolution ions from BG with increasing strontium substitution reduced erosion pit area on CaP-coated glass slides. This suggests that the strontium ions either inhibit osteoclast differentiation or prevent mature osteoclasts from eroding the coating. SrR has been previously shown to both inhibit osteoclast differentiation and resorption activities *in vitro* [24]. Recent studies have suggested that modulation of osteoprotegerin and RANKL expression, known regulators of osteoclast activity and resorption, are responsible for this effect [25]. Others, however, have shown that SrR inhibits osteoclast activity by preventing the formation of the ruffled border region and clear zone necessary for sealing and erosion pit formation [5, 26]. Our data show that dissolution ions from strontium-containing BG did not prevent erosion pit formation to the same extent as the bisphosphonate pamidronate. Bisphosphonates act by disrupting the mevalonate pathway, disturbing osteoclast cytoskeleton formation and their ability to form a ruffled border region (reviewed in [27]). The mechanism by which strontium ions prevent erosion pit formation in the present study is not clear, but inhibition of differentiation and disruption of cytoskeletal elements are two possibilities.

Substituting strontium for calcium in BG significantly decreased TRAP activity in cultures treated with their dissolution ions. ICP-OES demonstrated that DMEM cell culture medium treated with 100%-substituted BG had a strontium concentration of 84 ppm which is similar to the concentration of strontium in cultures supplemented with 1mM Sr^{2+} (88 ppm) (as either $SrCl_2 \cdot 6H_2O$ or SrR), which has also been shown to prevent osteoclast-mediated erosion of dentine slices [26] and apatite-coated matrices [5]. This level, however, is still higher than that in the serum of patients treated with SrR (0.12 mM Sr^{2+} or ~10 ppm [3]). Decreased TRAP activity, however, did not

come as a result of cell death. While there is evidence of an apoptotic effect of SrR on osteoclasts [28, 29], we could not detect significant differences in the number of cells in different groups at any time point. This is in agreement with studies which have shown that SrR does not affect attachment to or viability of osteoclasts on bone slices, but does reduce osteoclast differentiation and resorption activity [24]. Hurtel-Lemaire, *et al.* have shown that SrR does induce osteoclast apoptosis, however, this was only evident at Sr^{2+} concentrations of 9 mM and higher, far more than that tested here and more than order of magnitude higher than that present in the serum of patients treated with SrR [29].

No addition of strontium to cell culture media by dissolution ions from BG reduced TRAP activity to the same extent as pamidronate. This is in keeping with results from a recent clinical trial that demonstrated a more subtle bone remodelling effect of SrR as compared to other osteoporosis treatments [30]. Parathyroid hormone (PTH) treatment enhances bone mineral density by both increasing bone formation and resorption. Anti-catabolic agents such as bisphosphonates, on the other hand, inhibit bone turnover, preserving the existing skeletal microarchitecture. Because of its mode of action, there has been concern that long-term treatment with bisphosphonates may 'freeze' bone, prohibiting normal repair processes. Recent reports of osteonecrosis in the jaw of patients treated with bisphosphonates seem to confirm this [31]. Neither PTH nor bisphosphonate treatments, however, address the lost bone remodelling balance inherent to osteoporosis. Bone regeneration therapies involving strontium may be preferred in this regard as they prevent osteoclast resorption without diminishing bone mineralisation and formation.

Strontium-substituted BG not only stimulated increased osteoblast metabolic activity and inhibited osteoclast differentiation and resorption *via* their dissolution ions, but also promoted osteoblast proliferation and ALP activity while directly in contact with cells. This is in agreement with studies on standard BG which have been shown to stimulate the expression of a number of osteogenic genes (reviewed in [32]), although the mechanism by which ions released from BG act on cells remains unclear. There is evidence that strontium's effect is mediated by cation-sensing receptors other than the calcium-sensing receptor (CaSR) [33, 34]. This is supported by data demonstrating that neither calcium nor sodium ranelate had the same effects as their strontium counterpart [23, 26]. However, this has been refuted by other studies which have demonstrated that strontium's effect is CaSR dependent [25, 29], although an effect mediated by a combination of receptors cannot be ruled out.

Osteoclasts on BG discs assumed typical resorption morphologies, which was in contrast to non-resorbing morphologies noted in osteoclasts on tissue culture plastic. Previous studies have demonstrated that only when adherent to apatite do osteoclasts assume typical apico-basal polarisation and form sealing rings [35] suggesting that the BG discs examined in this study are sufficient to elicit a normal bone remodelling response. Our data also suggest that ions dissolved from BG are not sufficient on their own to promote a resorption phenotype as osteoclasts cultured on tissue culture plastic in the presence of BG dissolution ions maintained flattened non-resorbing morphologies. This is in agreement with previous studies which have shown that microenvironmental cues alone do not trigger osteoclast polarisation and sealing zone formation [35].

5. Conclusions

Strontium can be substituted for calcium in BG, creating a biomaterial that increases osteoblast proliferation and ALP activity, and inhibits TRAP activity and osteoclast-mediated resorption of CaP films, similarly to the osteoporosis drug SrR. As BG are a well-studied biomaterial system capable of controlled ion release and are deliverable as particulate, scaffolds or bulk material, substitution of strontium for calcium in BG may be an effective strategy for creating materials for bone repair/regeneration therapies.

6. Acknowledgements

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Figure captions

Figure 1: Elemental concentrations of Ca, P, Si and Sr as determined by ICP-OES in cell culture media over 4 hours incubation with BG particles in which 0, 10, 50 or 100% of the calcium was substituted for with strontium. Dissolution behaviour into **a**. RPMI 1640 and **b**. DMEM.

Figure 2: Normalised MTT activity of Saos-2 human osteosarcoma cells treated with dissolution ions from standard BG and BG in which increasing proportions of calcium were substituted for with strontium. Data represent mean + standard deviation and are normalised to the mean value of controls after 1 day in culture. * indicates significantly higher MTT activity in the indicated group compared to the control group at the same time point. † compared to 0%, ‡ compared to 10%, and § compared to 50% (all at the same time point).

Figure 3: Representative images of erosion pits (bright) formed on calcium phosphate-coated chamber slides by RAW264.7 mouse monocytes cultured for 4 days in the presence of either **a**. RANKL alone or **b**. RANKL combined with 1 μM pamidronate, a drug known to prevent osteoclast-mediated bone resorption *in vivo*. Scale bar in **a** and **b** is 1 mm. **c**. Mean erosion pit area (± standard deviation) in calcium phosphate-coated chamber slides formed by RAW264.7 cells treated with RANKL and culture media containing dissolution ions from standard and strontium-substituted BG (■). Cultures supplemented with RANKL alone (CNT ○), 1 μM pamidronate (PAM ○) and 1 mM $SrCl_2$ (▲) are shown as controls.

Figure 4: **a**. Representative epifluorescense micrograph showing large multinucleated osteoclasts with typical podosome belts (arrows) in cultures of RAW264.7 mouse monocytes after 4 days treatment with RANKL and dissolution ions from 50% strontium-substituted BG. **b**. Epifluorescense micrograph of RAW264.7 treated with RANKL and 1 μM pamidronate in which no multinucleated osteoclasts are visible. Red = phalloidin (actin), Blue = DAPI (nuclei). Scale bar in **a** and **b** is 200 μm. **c**. Total TRAP activity (mean + standard deviation) expressed as nmoles p-nitrophenyl liberated per minute in the presence of 20 mM tartrate in RAW264.7 differentiated in BG dissolution ion media or with 1μM pamidronate. All cultures were supplemented with 20 ng/mL RANKL. Pamidronate significantly reduced total TRAP activity at all time points compared to all other groups (#). After 4 days, RAW264.7 treated with 100% strontium-substituted BG had significantly reduced TRAP activity compared to all other glass treated groups and CNT (*compared to CNT at same time point; † compared to 0%; ‡10%; §50%). After 6 days, cells treated with 10, 50 and 100% strontium-substituted BG had significantly lower TRAP activity when compared to 0% (†). **d**. Total number of cells in cultures of RAW264.7 monocytes treated with dissolution ions from BG or pamidronate. There were no significant differences between the number of cells detected in any group.

Figure 5: **a**. Total number of cells on BG discs after 1, 7 and 14 days in culture as determined by measuring lactate dehydrogenase activity. After 7 days in culture, there were significantly more cells on 100% substituted BG discs than on 0%, and after 14 days in culture there were significantly more cells on 50 and 100% strontium-substituted BG discs compared to either 0 or 10% († significantly more cells compared to 0% at the same time point, ‡ compared to 10%). **b**. Total alkaline phosphatase (ALP) activity (mean + standard deviation) in Saos-2 cells on 0% strontium BG discs and BG in which 10, 50 or 100% of the calcium was substituted for with strontium. After 7 days in culture, cells on 100% strontium-substituted BG discs had significantly higher total ALP activity than those on 0% or 10% substituted BG discs. After 2 weeks in culture, cells on discs in which 50 or 100% of the calcium had been substituted for with strontium had significantly higher total ALP activity than those on 0% or 10% strontium-substituted BG discs († significantly greater total ALP activity compared to 0% at the same time point, ‡ compared to 10%).

Figure 6: SEM image of osteoclast on 0% strontium-substituted BG disc after 4 days in culture. A large osteoclast with a typical apico-basal polarised resorbing morphology (black arrow) is visible as are many undifferentiated monocytes (white arrows). Scale bar is 100 μm.

Supplementary Figure 1: Tartrate resistant acid phosphatase (TRAP) activity normalised to cell number in RAW264.7 monocytes differentiated for 2, 4 and 6 days in media containing dissolution ions from BG or 1μM pamidronate. All cultures were supplemented with RANKL. Pamidronate significantly reduced TRAP activity per cell (*). Although TRAP activity per cell decreased when cells were treated with dissolution ions made from BG with increasing strontium substitution for calcium, this trend was not significant.

Supplementary Figure 2: Alkaline phosphatase (ALP) activity normalised to cell number of Saos-2 human osteosarcoma cells on discs of standard BG and BG in which 10, 50 or 100% of the calcium was substituted for with strontium. After 14 days in culture, cells on 50 and 100% substituted BG had significantly higher ALP activity per cell than cells on 0% substituted BG.

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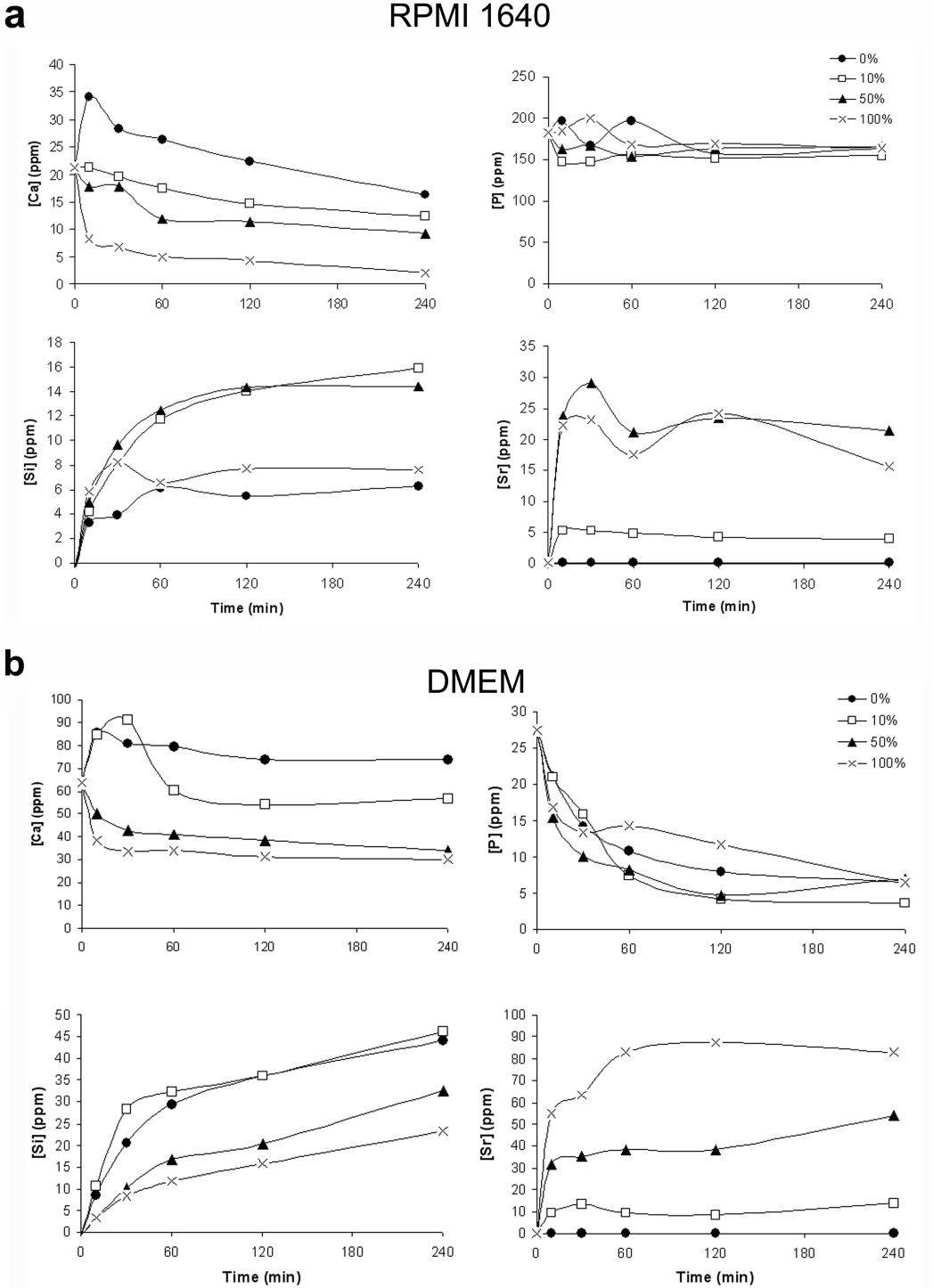
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[P] (ppm)
[Si] (ppm)
[Sr] (ppm)
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Time (min)
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10%
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b
DMEM
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[Sr] (ppm)
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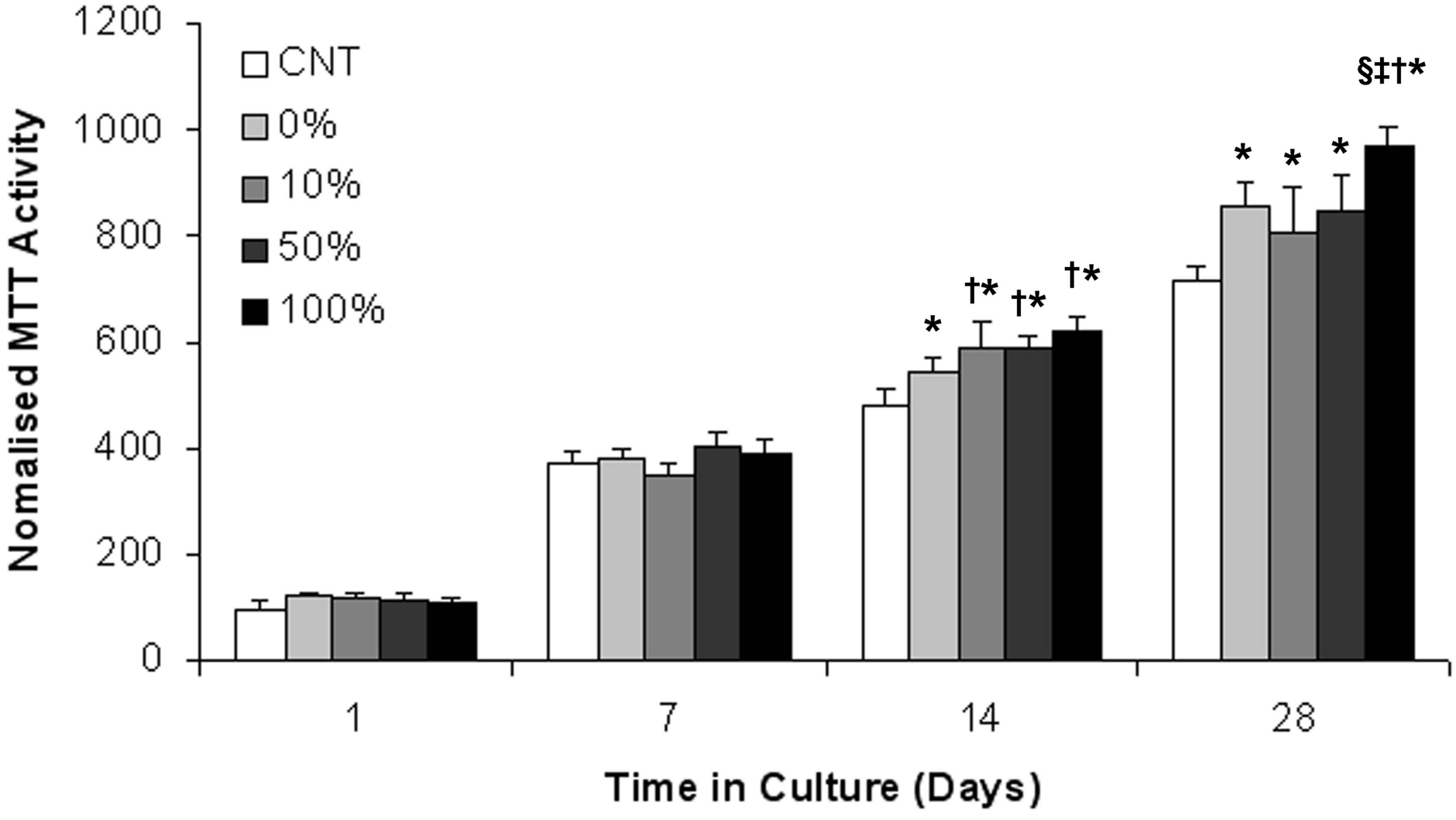
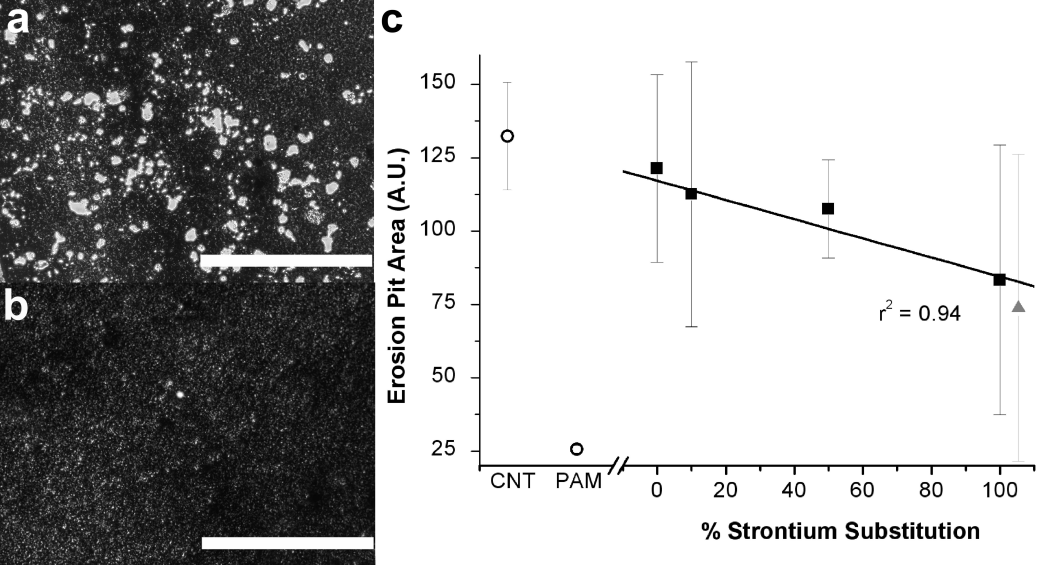
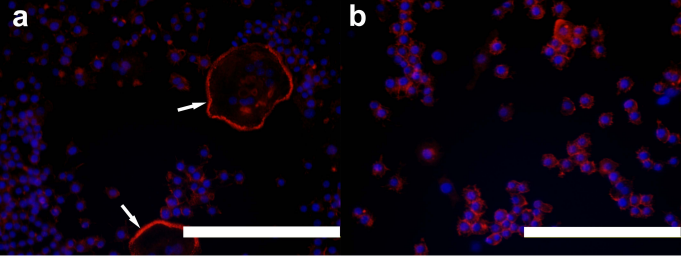
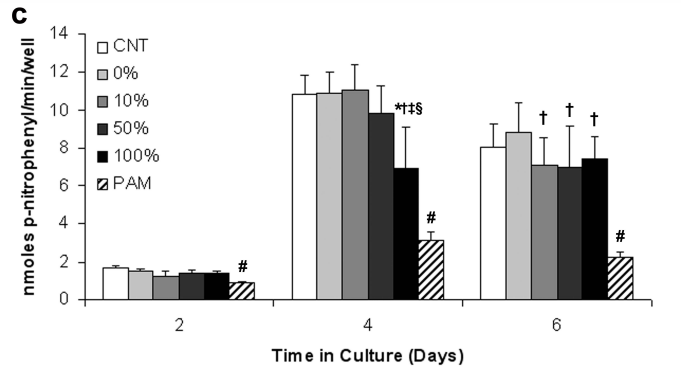
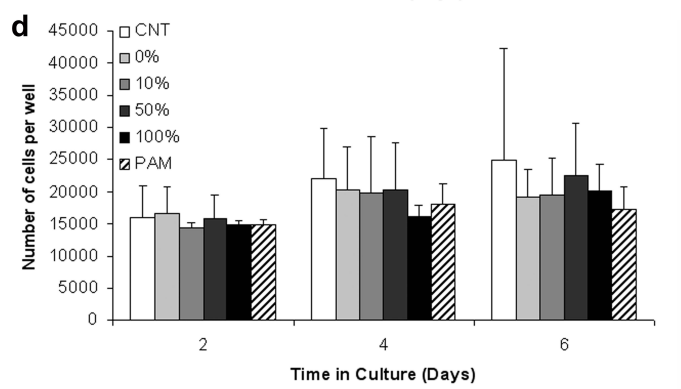
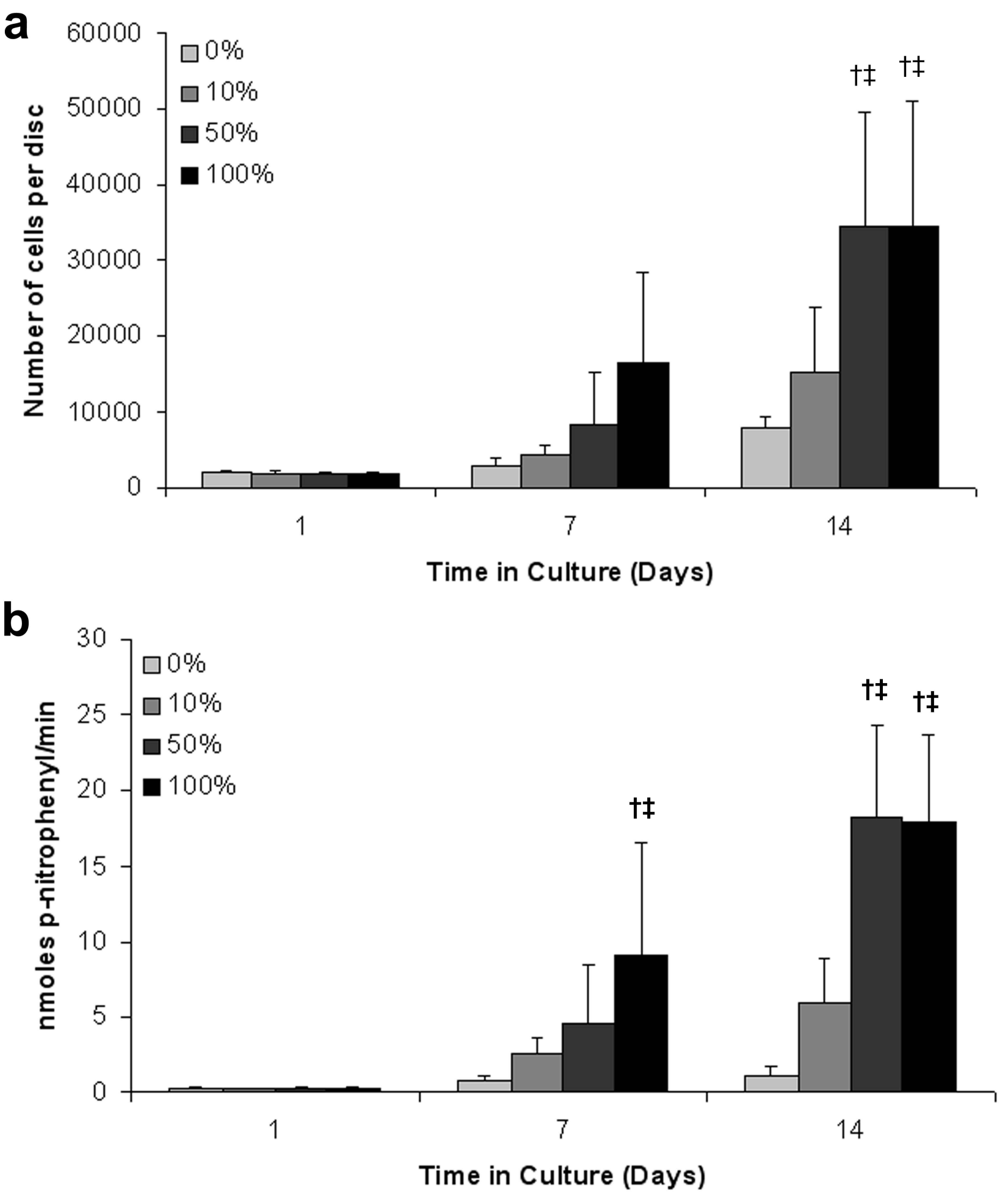

1200
1000
800
600
400
200
0
CNT
0%
10%
50%
100%
Nomalised MTT Activity
*
†*
†*
†*
*
*
*
§‡†*
1
7
14
28
Time in Culture (Days)


a
b
c
Erosion Pit Area (A.U.)
150
125
100
75
50
25
CNT
PAM
0
20
40
60
80
100
% Strontium Substitution
r2 = 0.94


a
b


c
nmoles p-nitrophenyl/min/well
14
12
10
8
6
4
2
0
CNT
0%
10%
50%
100%
PAM
*†‡§
†
†
†
#
#
#
2
4
6
Time in Culture (Days)


d
Number of cells per well
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
CNT
0%
10%
50%
100%
PAM
2
4
6
Time in Culture (Days)


a
60000
50000
40000
30000
20000
10000
0
0%
10%
50%
100%
Number of cells per disc
†‡
†‡
1
7
14
Time in Culture (Days)
b
30
25
20
15
10
5
0
0%
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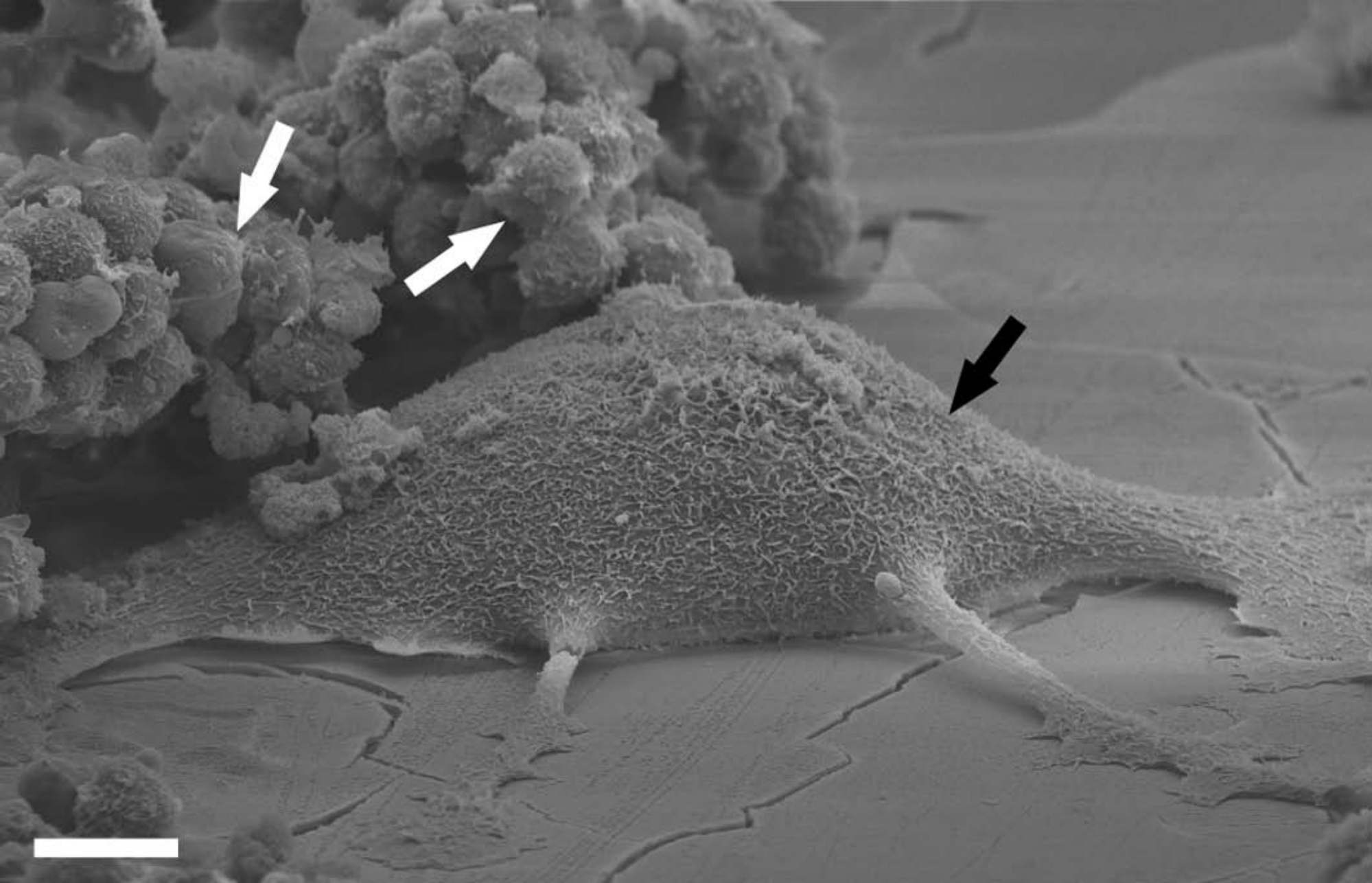
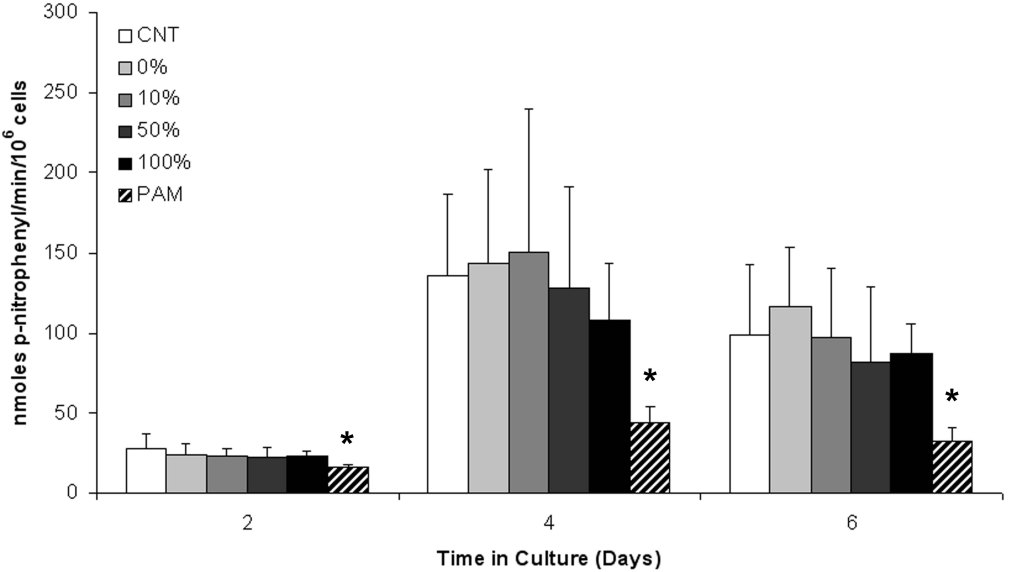
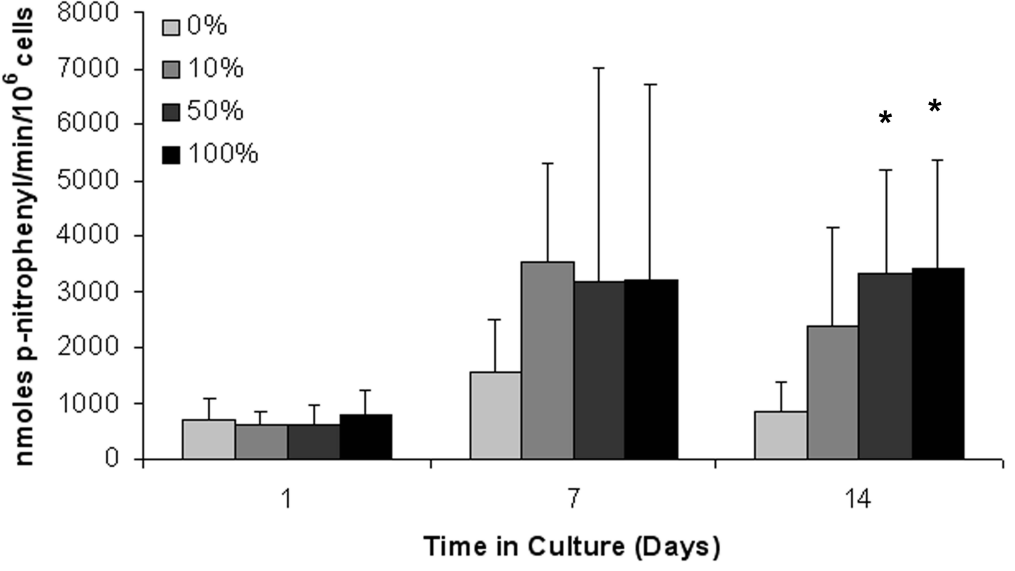
50%
100%
nmoles p-nitrophenyl/min
†‡
†‡
†‡
1
7
14
Time in Culture (Days)




300
250
200
150
100
50
0
CNT
0%
10%
50%
100%
PAM
nmoles p-nitrophenyl/min/10^6 cells
*
*
*
2
4
6
Time in Culture (Days)


8000
7000
6000
5000
4000
3000
2000
1000
0
nmoles p-nitrophenyl/min/10^6 cells
0%
10%
50%
100%
*
*
1
7
14
Time in Culture (Days)